

Fungal cell wall degrading enzyme.**Field of invention**

The present invention relates to a fungal enzyme having lysozyme activity, a nucleotide sequence encoding the lysozyme, a nucleic acid construct comprising the nucleotide sequence, methods of producing the lysozyme, as well as uses of the lysozyme.

Background of the invention

Lysozyme is a O-glycosyl hydrolase produced as a defensive mechanism against bacteria by many organisms. The enzyme causes the hydrolysis of bacterial cell walls by cleaving the glycosidic bonds of peptidoglycan; an important structural molecule in bacteria. After having their cell walls weakened by lysozyme action, bacterial cells lyse resulting from osmotic pressure.

Lysozyme occurs in many organisms such as viruses, plants, insects, birds, reptiles and mammals. In mammals, Lysozyme has been isolated from nasal secretions, saliva, tears, intestines, urine and milk. The enzyme cleaves the glycosidic bond between carbon number 1 of N-acetylmuramic acid and carbon number 4 of N-acetyl-D-glucosamine. In vivo, these two carbohydrates are polymerized to form the cell wall polysaccharide.

Bacteria against which Lysozyme has shown effectiveness include *Clostridium butyricum*, *Clostridium sporogenes*, *Clostridium tyrobutyricum*, *Listeria monocytogenes*.

The lytic action of egg white lysozyme protein is presently being exploited in two markets.

In cheese production, Lysozyme has been found effectively destructive on the vegetative forms of Clostridia bacteria and specifically *Clostridium tyrobutyricum*. These bacteria have been found to survive the normal heat treatment of milk used in the production of cheese and later propagate to cause late blowing. Late blowing is the formation of gasses during butyric fermentation occurring in the course of cheese maturation. The effects of the unwanted gas formation can be: faults in texture through the development of irregular-shaped eyes; or obvious undesirable tastes and smells; and finally, the cheese block may completely break apart. With the use of Lysozyme in the milk culture, the production and curing of the cheese may be carried out without concern for butyric fermentation causing late blowing effects. This has led to the wide-spread use of Lysozyme in European cheese production, particularly in the manufacture of medium and long-term ripening cheese. The recommended dosage is

0.2 lbs/1000 gallons milk. The lysozyme must be added after the last heat treatment due to heat instability, and as early as possible before rennet is added due to protease sensitivity.

In pharmacology, Lysozyme's natural function in biological liquids is to attack bacteria foreign 5 to the body. Many bacteria that invade the body through any typical route-eyes, mouth, nose and wounds are challenged with the human immunological system, of which Lysozyme is a critical part. This anti-infectious activity has been exploited in the pharmaceutical industry by producing pharmaceutical tablets and capsules containing this egg white derived protein. It is also an important component in eye drops, toothpaste and throat lozenges. Potential uses for 10 Lysozyme are varied. Successful results have been determined in cancer research and veterinary applications. As a food preservative, Lysozyme is a natural, organic alternative to many potential carcinogens. Lysozyme's use in baby food applications as well as in animal feed has shown positive results. The potential for this egg white derived enzyme is diverse and research and development on future applications is ongoing.

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A GH25 lysozyme has been reported from Chalaropsis (Felsch JW, Ingagami T, and Hash JH. (1975) The N,O-Diacetylmuramidase of Chalaropsis species; V The complete amino acid sequence. JBC. 250:10 pp 3713-3720).

20 Hen egg white lysozyme which is the primary product available on the commercial market, does not cleave N,6-O-diacetylmuramidase in e.g. *Streptococcus aureus* cell walls and is thus unable to lyse this important human pathogen among others.

New polypeptides having lysozyme activity is therefore desired.

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Summary of the invention

The inventors of the present invention have isolated a fungal gene/cDNA encoding an enzyme having lysozyme activity. Such a gene/cDNA encoding the enzyme has to our knowledge not previously been described.

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In a first aspect the present invention therefore relates to a fungal polypeptide having lysozyme activity and belonging to the GH25 family.

In a second aspect the present invention relates to a polynucleotide having a nucleotide 35 sequence which encodes for the polypeptide of the invention.

Definitions

Prior to a discussion of the detailed embodiments of the invention, a definition of specific terms related to the main aspects of the invention is provided.

5 **Abbreviations:** GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid.

Substantially pure polypeptide: In the present context, the term "substantially pure polypeptide" means a polypeptide preparation which contains at the most 10% by weight of other polypeptide material with which it is natively associated (lower percentages of other 10 polypeptide material are preferred, e.g. at the most 8% by weight, at the most 6% by weight, at the most 5% by weight, at the most 4%, at the most 3% by weight, at the most 2% by weight, at the most 1% by weight, and at the most ½% by weight). Thus, it is preferred that the substantially pure polypeptide is at least 92% pure, i.e. that the polypeptide constitutes at least 92% by weight of the total polypeptide material present in the preparation, and higher 15 percentages are preferred such as at least 94% pure, at least 95% pure, at least 96% pure, at least 96% pure, at least 97% pure, at least 98% pure, at least 99% pure, and at the most 99.5% pure. The polypeptides disclosed herein are preferably in a substantially pure form. In particular, it is preferred that the polypeptides disclosed herein are in "essentially pure form", i.e. that the polypeptide preparation is essentially free of other polypeptide material with 20 which it is natively associated. This can be accomplished, for example, by preparing the polypeptide by means of well-known recombinant methods. Herein, the term "substantially pure polypeptide" is synonymous with the terms "isolated polypeptide" and "polypeptide in isolated form".

25 **Lysozyme activity:** The term lysozyme activity is defined herein as an O-glycosyl hydrolase, which catalyses the hydrolysis of the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. Lysozymes cleave the glycosidic bond between certain residues in mucopolysaccharides and mucopeptides of bacterial cell walls, resulting in bacteriolysis. Lysozyme belongs to the enzyme class EC 3.2.1.17.

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Identity: In the present context, the homology between two amino acid sequences or between two nucleotide sequences is described by the parameter "identity".

For purposes of the present invention, the degree of identity between two amino acid sequences is determined by a Needleman-Wunsch alignment, useful for both protein 35 and DNA alignments. For protein alignments the default scoring matrix used is BLOSUM50, and the penalty for the first residue in a gap is -12, while the penalty for additional residues in

a gap is -2. The alignment may be made with the Align software from the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448; and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98).

5 The degree of identity between two nucleotide sequences may be determined using the same algorithm and software package as described above using the identity matrix as the default scoring matrix. The penalty for the first residue in a gap is -16, while the penalty for additional residues in a gap is -4.

10 Fragment: When used herein, a "fragment" of SEQ ID NO:2 is a polypeptide retaining its lysozyme activity but having one or more amino acids deleted from the amino and/or carboxyl terminus of this amino acid sequence. Preferably, a fragment contains at the most 208 amino acid residues, e.g., amino acids 20 to 228 of SEQ ID NO:2.

15 Substantially pure polynucleotide: The term "substantially pure polynucleotide" as used herein refers to a polynucleotide preparation, wherein the polynucleotide has been removed from its natural genetic milieu, and is thus free of other extraneous or unwanted coding sequences and is in a form suitable for use within genetically engineered protein production systems. Thus, a substantially pure polynucleotide contains at the most 10% by weight of 20 other polynucleotide material with which it is natively associated (lower percentages of other polynucleotide material are preferred, e.g. at the most 8% by weight, at the most 6% by weight, at the most 5% by weight, at the most 4% at the most 3% by weight, at the most 2% by weight, at the most 1% by weight, and at the most ½% by weight). A substantially pure polynucleotide may, however, include naturally occurring 5' and 3' untranslated regions, such 25 as promoters and terminators. It is preferred that the substantially pure polynucleotide is at least 92% pure, i.e. that the polynucleotide constitutes at least 92% by weight of the total polynucleotide material present in the preparation, and higher percentages are preferred such as at least 94% pure, at least 95% pure, at least 96% pure, at least 96% pure, at least 97% pure, at least 98% pure, at least 99% pure, and at the most 99.5% pure. The 30 polynucleotides disclosed herein are preferably in a substantially pure form. In particular, it is preferred that the polynucleotides disclosed herein are in "essentially pure form", i.e. that the polynucleotide preparation is essentially free of other polynucleotide material with which it is natively associated. Herein, the term "substantially pure polynucleotide" is synonymous with the terms "isolated polynucleotide" and "polynucleotide in isolated form".

Modification(s): In the context of the present invention the term "modification(s)" is intended to mean any chemical modification of the polypeptide consisting of the amino acid sequence shown as amino acids 1 to 233 of SEQ ID NO:2 as well as genetic manipulation of the DNA encoding that polypeptide. The modification(s) can be replacement(s) of the amino acid side 5 chain(s), substitution(s), deletion(s) and/or insertions(s) in or at the amino acid(s) of interest.

Artificial variant: When used herein, the term "artificial variant" means a polypeptide having lysozyme activity, which has been produced by an organism which is expressing a modified gene as compared to SEQ ID NO:1. The modified gene, from which said variant is produced 10 when expressed in a suitable host, is obtained through human intervention by modification of the nucleotide sequence disclosed in SEQ ID NO:1.

cDNA: The term "cDNA" when used in the present context, is intended to cover a DNA molecule which can be prepared by reverse transcription from a mature, spliced, mRNA 15 molecule derived from a eukaryotic cell. cDNA lacks the intron sequences that are usually present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA and it goes through a series of processing events before appearing as mature spliced mRNA. These events include the removal of intron sequences by a process called splicing. When cDNA is derived from mRNA it therefore lacks intron sequences.

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Nucleic acid construct: When used herein, the term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature. The term nucleic acid construct is synonymous with the 25 term "expression cassette" when the nucleic acid construct contains the control sequences required for expression of a coding sequence of the present invention.

Control sequence: The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of a polypeptide of the present 30 invention. Each control sequence may be native or foreign to the nucleotide sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with 35 linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleotide sequence encoding a polypeptide.

Operably linked: The term "operably linked" is defined herein as a configuration in which a control sequence is appropriately placed at a position relative to the coding sequence of the DNA sequence such that the control sequence directs the expression of a polypeptide.

5 Coding sequence: When used herein the term "coding sequence" is intended to cover a nucleotide sequence, which directly specifies the amino acid sequence of its protein product. The boundaries of the coding sequence are generally determined by an open reading frame, which usually begins with the ATG start codon. The coding sequence typically include DNA, cDNA, and recombinant nucleotide sequences.

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Expression: In the present context, the term "expression" includes any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

15 Expression vector: In the present context, the term "expression vector" covers a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of the invention, and which is operably linked to additional segments that provide for its transcription.

20 Host cell: The term "host cell", as used herein, includes any cell type which is susceptible to transformation with a nucleic acid construct. In case a bacterial cell is used as the host cell it will not be suitable for expression of the fungal polypeptide of the invention, however, a bacterial host cell may be used for cloning purposes.

25 The terms "polynucleotide probe", "hybridization" as well as the various stringency conditions are defined in the section entitled "Polypeptides Having Lysozyme Activity".

Detailed description of the invention

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Polypeptides having lysozyme activity

The polypeptide of the invention was isolated from a fungus, more precisely from a *Pycnoporus cinnabarinus* a synonym is *Trametes cinnabrina* in a screening for highly secreted polypeptides and the function of the polypeptide determined.

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In a first embodiment, the present invention relates to fungal polypeptide having lysozyme activity and belonging to the GH25 family. The EC 3.2.1.17 enzyme activity classification encompasses enzymes that perform the hydrolysis of the 1,4-beta-linkages between N-acetyl-D-glucosamine and N-acetylmuramic acid in peptidoglycan heteropolymers of the 5 prokaryotes cell wall. An enzyme may have one substrate specificity (for example N-acetylmuramic acid) without the other (N-acetyl-D-glucosamine in this case).

The GH25 family is a classification of enzymes according to the Henrissat glycosyl hydrolase family classification (Henrissat B., A classification of glycosyl hydrolases based on amino- 10 acid sequence similarities. Biochem. J. 280:309-316(1991); Henrissat B., Bairoch A. New families in the classification of glycosyl hydrolases based on amino- acid sequence similarities. Biochem. J. 293:781-788(1993); Henrissat B., Bairoch A. Updating the sequence-based classification of glycosyl hydrolases. Biochem. J. 316:695-696(1996); Davies G., Henrissat B. Structures and mechanisms of glycosyl hydrolases. Structure 3:853- 15 859(1995)).

Besides GH25, other enzyme hydrolase families with lysozyme (EC 3.2.1.17) activity are GH22, GH23 and GH24. Family GH22, or lysozyme C includes hen egg white lysozyme and the stomach lysozymes of ruminants. This family is the most extensively studied of the four 20 families. GH23 commonly known as lysozyme G is represented by Goose and a number of less well studied phage and bacterial sequences from genome sequencing projects. GH24 includes notably phage T4 and other phage and bacterial lysozymes. Research by Seo et al. 2003 (Seo HJ; Kitaoka M; Ohmiya K; Hayashi K., (2003) Substrate specificity of the N,6-O-diacetylmuramidase from *Streptomyces globisporus*. Journal of Bioscience and 25 Bioengineering, Vol. 95 (3) pp. 313-316) have shown that, at least in the case of *Streptomyces globisporus* GH25 enzyme, a different activity spectra was observed compared to hen egg white lysozyme GH22. The *Streptomyces* enzyme possesses both N-acetylmuramidase and N,6-O-diacetylmuramidase activity. This allowed the *Streptomyces globisporus* enzyme to degrade *Staphylococcus aureus* cell walls, which are highly 30 acetylated. This activity may be extrapolatable to other lysozyme enzymes in this family such as the lysozyme GH25 of the invention.

In a further embodiment the present invention relates to polypeptides where the polypeptides comprises, preferably consists of, an amino acid sequence which has a degree of identity to 35 amino acids 1 to 233 of SEQ ID NO:2 of at least 65%, preferably at least 70%, e.g. at least 75%, more preferably at least 80%, such as at least 85%, even more preferably at least 90%,

most preferably at least 95%, e.g. at least 96%, such as at least 97%, and even most preferably at least 98%, such as at least 99% (hereinafter "homologous polypeptides"). In an interesting embodiment, the amino acid sequence differs by at the most ten amino acids (e.g. by ten amino acids), in particular by at the most five amino acids (e.g. by five amino acids),
5 such as by at the most four amino acids (e.g. by four amino acids), e.g. by at the most three amino acids (e.g. by three amino acids) from amino acids 1 to 233 of SEQ ID NO:2. In a particular interesting embodiment, the amino acid sequence differs by at the most two amino acids (e.g. by two amino acids), such as by one amino acid from amino acids 1 to 233 of SEQ ID NO:2.

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Preferably, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO:2; an allelic variant thereof; or a fragment thereof that has lysozyme activity. In another preferred embodiment, the polypeptide of the present invention comprises amino acids 1 to 233 of SEQ ID NO:2. In a further preferred embodiment, the polypeptide 15 consists of amino acids 1 to 233 of SEQ ID NO:2.

Polypeptides of the invention may be a wild type lysozyme identified and isolated from a natural source. Such wild-type polypeptides may be specifically identified e.g. by a bioinformatics approach, which is one method that can be used to identify lysozyme 20 candidates. Briefly GH25 lysozyme candidates can be directly identified in a genome or cDNA sequencing project by using a known lysozyme of family GH25 as the search sequence. Another method is to compare all of the unique DNA fragments (contigs) a standard homology search program such as WU-BlastX (2.0a19MP_WashU).

25 Furthermore, the polypeptide of the invention may be prepared by the DNA shuffling technique, such as described in J.E. Ness et al. *Nature Biotechnology* 17, 893-896 (1999). Moreover, the polypeptide of the invention may be an artificial variant which comprises, preferably consists of, an amino acid sequence that has at least one substitution, deletion and/or insertion of an amino acid as compared to amino acids 1 to 233 of SEQ ID NO:2.
30 Such artificial variants may be constructed by standard techniques known in the art, such as by site-directed/random mutagenesis of the polypeptide comprising the amino acid sequence shown as amino acids 1 to 233 of SEQ ID NO:2. In one embodiment of the invention, amino acid changes (in the artificial variant as well as in wild-type polypeptides) are of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding
35 and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a

small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids 5 (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine, valine and methionine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine and threonine). Amino acid substitutions which do not generally alter the specific activity are known in the art and are described, for 10 example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly as well as these in reverse.

In an interesting embodiment of the invention, the amino acid changes are of such a 15 nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may be performed, which improve the thermal stability of the polypeptide, which alter the substrate specificity, which changes the pH optimum, and the like.

Preferably, the number of such substitutions, deletions and/or insertions as 20 compared to amino acids 1 to 233 of SEQ ID NO:2 is at the most 10, such as at the most 9, e.g. at the most 8, more preferably at the most 7, e.g. at the most 6, such as at the most 5, most preferably at the most 4, e.g. at the most 3, such as at the most 2, in particular at the most 1.

In another embodiment, the present invention relates to polypeptides having 25 lysozyme activity which are encoded by nucleotide sequences which hybridize under very low stringency conditions, preferably under low stringency conditions, more preferably under medium stringency conditions, more preferably under medium-high stringency conditions, even more preferably under high stringency conditions, and most preferably under very high stringency conditions with a polynucleotide probe consisting of the complementary strand of 30 nucleotides 84 to 782 of SEQ ID NO:1 (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

The nucleotide sequence of SEQ ID NO:1 or a subsequence thereof, as well as the amino acid sequence of SEQ ID NO:2 or a fragment thereof, may be used to design a polynucleotide probe to identify, and clone DNA encoding polypeptides having lysozyme 35 activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the

genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, preferably at least 25, more preferably at least 35 nucleotides in length, such as at least 70 nucleotides in length. It is, 5 however, preferred that the polynucleotide probe is at least 100 nucleotides in length. For example, the polynucleotide probe may be at least 200 nucleotides in length, at least 300 nucleotides in length, at least 400 nucleotides in length or at least 500 nucleotides in length. Even longer probes may be used, e.g., polynucleotide probes which are at least 600 10 nucleotides in length, at least 700 nucleotides in length, at least 800 nucleotides in length, or at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P , ^{3}H , ^{35}S , biotin, or avidin).

Thus, a genomic DNA or cDNA library prepared from such other organisms may be screened for DNA which hybridizes with the probes described above and which encodes a 15 polypeptide having lysozyme activity. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to, and immobilized, on nitrocellulose or other suitable carrier materials. In order to identify a clone or DNA which is homologous with SEQ ID NO:1 the carrier material with the immobilized 20 DNA is used in a Southern blot.

For purposes of the present invention, hybridization indicates that the nucleotide sequence hybridizes to a labeled polynucleotide probe which hybridizes to the nucleotide sequence shown in SEQ ID NO:1 under very low to very high stringency conditions. Molecules to which the polynucleotide probe hybridizes under these conditions may be 25 detected using X-ray film or by any other method known in the art. Whenever the term "polynucleotide probe" is used in the present context, it is to be understood that such a probe contains at least 15 nucleotides.

In an interesting embodiment, the polynucleotide probe is the complementary strand of nucleotides 84 to 782 of SEQ ID NO:1.

30 In another interesting embodiment, the polynucleotide probe is the complementary strand of the nucleotide sequence which encodes the polypeptide of SEQ ID NO:2. In a further interesting embodiment, the polynucleotide probe is the complementary strand of SEQ ID NO:1. In a still further interesting embodiment, the polynucleotide probe is the complementary strand of the mature polypeptide coding region of SEQ ID NO:1.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as pre-hybridization and hybridization at 42°C in 5X SSPE, 1.0% SDS, 5X Denhardt's solution, 100 µg/ml sheared and denatured salmon sperm DNA, following standard Southern blotting procedures. Preferably, the long probes of at least 100

5 nucleotides do not contain more than 1000 nucleotides. For long probes of at least 100 nucleotides in length, the carrier material is finally washed three times each for 15 minutes using 2 x SSC, 0.1% SDS at 42°C (very low stringency), preferably washed three times each for 15 minutes using 0.5 x SSC, 0.1% SDS at 42°C (low stringency), more preferably washed three times each for 15 minutes using 0.2 x SSC, 0.1% SDS at 42°C (medium stringency),

10 even more preferably washed three times each for 15 minutes using 0.2 x SSC, 0.1% SDS at 55°C (medium-high stringency), most preferably washed three times each for 15 minutes using 0.1 x SSC, 0.1% SDS at 60°C (high stringency), in particular washed three times each for 15 minutes using 0.1 x SSC, 0.1% SDS at 68°C (very high stringency).

Although not particularly preferred, it is contemplated that shorter probes, e.g.

15 probes which are from about 15 to 99 nucleotides in length, such as from about 15 to about 70 nucleotides in length, may be also be used. For such short probes, stringency conditions are defined as prehybridization, hybridization, and washing post-hybridization at 5°C to 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proceedings of the National Academy of Sciences USA* 48:1390) in 0.9 M NaCl, 0.09 M Tris-

20 HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures.

For short probes which are about 15 nucleotides to 99 nucleotides in length, the carrier material is washed once in 6X SCC plus 0.1% SDS for 15 minutes and twice each for

25 15 minutes using 6X SSC at 5°C to 10°C below the calculated T_m .

Lysozyme Activity.

Several assays have been described for the determination of lysozyme activity. One assay described in J. Biochem. (H. Maeda, 1980, vol. 88 (4): 1185-1191) is based on either

30 fluorescense polarization or fluorescense intesity using fluorescein-labeled peptidoglycan as a substrate, which was obtained from *Micrococcus lysodeikticus*.

Another assay is based on the clearance of a *Micrococcus luteus* suspension caused by lysozyme and monitored at 450 nm and compared to a lysozyme standard of known activity. This assay was developed at the FIP Center for Standards, International

35 Commission on Pharmaceutical Enzymes, Harelbekestaat 72, B-9000 gent, Belgium. This

assay requires the use of lyophilized, viable *M. luteus* ATCC 4698 cells. This substrate is available from the Center for Standards as well as a detailed protocol.

Another assay, EnzChek® Lysozyme Assay Kit (E-22013), is available from Molecular Probes, and is based on a measure of lysozyme activity on *Micrococcus lysodeikticus* cell walls that are labeled with fluorescein.

Sources for Polypeptides having Lysozyme Activity

A polypeptide of the present invention is a fungal polypeptide. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth *et al.*, 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth *et al.*, 1995, *supra*).

In a particular embodiment, the fungal polypeptide is a yeast polypeptide. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, F.A., Passmore, S.M., and Davenport, R.R., eds, *Soc. App. Bacteriol. Symposium Series No. 9*, 1980).

The yeast polypeptide is in a particular embodiment a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide. In an interesting embodiment, the polypeptide is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis* or *Saccharomyces oviformis* polypeptide.

In another particular embodiment the fungal polypeptide of the invention is a filamentous fungal polypeptide such as an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, or *Trichoderma* polypeptide.

In another interesting embodiment, the polypeptide is an *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sارcochroum*, *Fusarium*

sporotrichioides, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*,

5 *Trichoderma reesei*, or *Trichoderma viride* polypeptide.

It will be understood that for the aforementioned species, the invention encompasses both the perfect and imperfect states, and other taxonomic equivalents, e.g., anamorphs, regardless of the species name by which they are known. Those skilled in the 10 art will readily recognize the identity of appropriate equivalents.

Strains of these species are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional 15 Research Center (NRRL).

Furthermore, such polypeptides may be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms from natural habitats are well known in the art. The nucleotide sequence may then be derived by similarly screening a 20 genomic or cDNA library of another microorganism. Once a nucleotide sequence encoding a polypeptide has been detected with the probe(s), the sequence may be isolated or cloned by utilizing techniques which are known to those of ordinary skill in the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

Polypeptides encoded by nucleotide sequences of the present invention also include 25 fused polypeptides or cleavable fusion polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleotide sequence (or a portion thereof) encoding another polypeptide to a nucleotide sequence (or a portion thereof) of the present invention. Techniques for producing fusion polypeptides are known in the art, and include ligating the 30 coding sequences encoding the polypeptides so that they are in frame and that expression of the fused polypeptide is under control of the same promoter(s) and terminator.

Polynucleotides and Nucleotide Sequences

The present invention also relates to polynucleotides having a nucleotide sequence 35 which encodes for a polypeptide of the invention. In particular, the present invention relates to polynucleotides consisting of a nucleotide sequence which encodes for a polypeptide of

the invention. In a particular embodiment, the nucleotide sequence is set forth in SEQ ID NO:1. In a more particular embodiment, the nucleotide sequence 139-724 is the mature polypeptide coding region of SEQ ID NO:1.

The present invention also encompasses polynucleotides having, preferably consisting of, 5 nucleotide sequences which encode a polypeptide consisting of the amino acid sequence of SEQ ID NO:2 or the mature polypeptide thereof, which differ from SEQ ID NO:1 by virtue of the degeneracy of the genetic code.

The present invention also relates to polynucleotides having, preferably consisting of, a subsequence of SEQ ID NO:1 which encode fragments of SEQ ID NO:2 that have 10 lysozyme activity. A subsequence of SEQ ID NO:1 is a nucleotide sequence encompassed by SEQ ID NO:1 except that one or more nucleotides from the 5' and/or 3' end have been deleted.

The present invention also relates to polynucleotides having, preferably consisting of, a modified nucleotide sequence which comprises at least one modification in the mature 15 polypeptide coding sequence of SEQ ID NO:1, and where the modified nucleotide sequence encodes a polypeptide which consists of amino acids 19 to 233 of SEQ ID NO:2.

The techniques used to isolate or clone a nucleotide sequence encoding a polypeptide are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the nucleotide sequences of the present 20 invention from such genomic DNA can be effected, e.g., by using the well known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural features. See, e.g., Innis *et al.*, 1990, *PCR: A Guide to Methods and Application*, Academic Press, New York. Other amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT) and nucleotide 25 sequence-based amplification (NASBA) may be used. The nucleotide sequence may be cloned from a strain of *Aspergillus*, or another or related organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the nucleotide sequence.

The nucleotide sequence may be obtained by standard cloning procedures used in 30 genetic engineering to relocate the nucleotide sequence from its natural location to a different site where it will be reproduced. The cloning procedures may involve excision and isolation of a desired fragment comprising the nucleotide sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into a host cell where multiple copies or clones of the nucleotide 35 sequence will be replicated. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

The present invention also relates to a polynucleotide having, preferably consisting of, a nucleotide sequence which has at least 65% identity with nucleotides 84 to 782 of SEQ ID NO:1. Preferably, the nucleotide sequence has at least 70% identity, e.g. at least 80% identity, such as at least 90% identity, more preferably at least 95% identity, such as at least 5 96% identity, e.g. at least 97% identity, even more preferably at least 98% identity, such as at least 99% with nucleotides 84 to 782 of SEQ ID NO:1. Particularly, the nucleotide sequence encodes a polypeptide having lysozyme activity. The degree of identity between two nucleotide sequences is determined as described previously (see the section entitled "Definitions").

10 Modification of a nucleotide sequence encoding a polypeptide of the present invention may be necessary for the synthesis of a polypeptide, which comprises an amino acid sequence that has at least one substitution, deletion and/or insertion as compared to amino acids 1 to 233 of SEQ ID NO:2. These artificial variants may differ in some engineered way from the polypeptide isolated from its native source, e.g., variants that differ in specific 15 activity, thermostability, pH optimum, or the like.

It will be apparent to those skilled in the art that such modifications can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acid residues essential to the activity of the polypeptide encoded by the nucleotide sequence of the invention, and therefore preferably not subject to modification, 20 such as substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (see, e.g., Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, mutations are introduced at every positively charged residue in the molecule, and the resultant mutant molecules are tested for lysozyme activity (as described in the examples) to identify amino acid residues 25 that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of the three-dimensional structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photoaffinity labelling (see, e.g., de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *Journal of Molecular Biology* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Letters* 309: 59-64).

30 Moreover, a nucleotide sequence encoding a polypeptide of the present invention may be modified by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the polypeptide encoded by the nucleotide sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme.

The introduction of a mutation into the nucleotide sequence to exchange one 35 nucleotide for another nucleotide may be accomplished by site-directed mutagenesis using any of the methods known in the art. Particularly useful is the procedure, which utilizes a

supercoiled, double stranded DNA vector with an insert of interest and two synthetic primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, extend during temperature cycling by means of *Pfu* DNA polymerase. On incorporation of the primers, a mutated plasmid containing staggered nicks 5 is generated. Following temperature cycling, the product is treated with *DpnI* which is specific for methylated and hemimethylated DNA to digest the parental DNA template and to select for mutation-containing synthesized DNA. Other procedures known in the art may also be used. For a general description of nucleotide substitution, see, e.g., Ford *et al.*, 1991, *Protein Expression and Purification 2*: 95-107.

10 The present invention also relates to a polynucleotide having, preferably consisting of, a nucleotide sequence which encodes a polypeptide having lysozyme activity, and which hybridizes under very low stringency conditions, preferably under low stringency conditions, more preferably under medium stringency conditions, more preferably under medium-high stringency conditions, even more preferably under high stringency conditions, and most 15 preferably under very high stringency conditions with a polynucleotide probe consisting of the complementary strand of nucleotides 84 to 782 of SEQ ID NO:1.

As will be understood, details and particulars concerning hybridization of the nucleotide sequences will be the same or analogous to the hybridization aspects discussed in the section entitled "Polypeptides having Lysozyme Activity" herein.

20

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a nucleotide sequence of the present invention operably linked to one or more control sequences that 25 direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

A nucleotide sequence encoding a polypeptide of the present invention may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the nucleotide sequence prior to its insertion into a vector may be desirable or necessary 30 depending on the expression vector. The techniques for modifying nucleotide sequences utilizing recombinant DNA methods are well known in the art.

The control sequence may be an appropriate promoter sequence, a nucleotide sequence which is recognized by a host cell for expression of the nucleotide sequence. The promoter sequence contains transcriptional control sequences, which mediate the 35 expression of the polypeptide. The promoter may be any nucleotide sequence which shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid

promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained 5 from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, and *Fusarium oxysporum* trypsin-like protease (WO 10 96/00787), as well as the NA2-tpi promoter (a hybrid of the promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate isomerase), and mutant, truncated, and hybrid promoters thereof.

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), 15 *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, Yeast 8: 423-488.

The control sequence may also be a suitable transcription terminator sequence, a 20 sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleotide sequence encoding the polypeptide. Any terminator which is functional in the host cell of choice may be used in the present invention.

Preferred terminators for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* 25 anthranilate synthase, *Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease.

Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful 30 terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a suitable leader sequence, a non-translated region of an mRNA which is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleotide sequence encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used in the present 35 invention.

Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, 5 *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3' terminus of the nucleotide sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA.

10 Any polyadenylation sequence which is functional in the host cell of choice may be used in the present invention.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Fusarium oxysporum* trypsin-like protease, and 15 *Aspergillus niger* alpha-glucosidase.

Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Molecular Cellular Biology* 15: 5983-5990.

The control sequence may also be a signal peptide coding region that codes for an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded 20 polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleotide sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region which is foreign to the coding sequence. The foreign signal peptide coding 25 region may be required where the coding sequence does not naturally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to enhance secretion of the polypeptide. However, any signal peptide coding region which directs the expressed polypeptide into the secretory pathway of a host cell of choice may be used in the present 30 invention.

The signal peptide coding region is nucleotides 84 to 138 of SEQ ID NO:1 which encode amino acids 1 to 18 of SEQ ID NO:2.

Effective signal peptide coding regions for filamentous fungal host cells are the signal peptide coding regions obtained from the genes for *Aspergillus oryzae* TAKA amylase, 35 *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Rhizomucor miehei* aspartic proteinase, *Humicola insolens* cellulase, and *Humicola lanuginosa* lipase.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding regions are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a propeptide coding region that codes for an 5 amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide.

Where both signal peptide and propeptide regions are present at the amino terminus 10 of a polypeptide, the propeptide region is positioned next to the amino terminus of a polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

It may also be desirable to add regulatory sequences which allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of 15 regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in yeast include the ADH2 system or GAL1 system, which may be used. In filamentous fungi, the TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used as 20 regulatory sequences. Other examples of regulatory sequences are those which allow for gene amplification. In eukaryotic systems, these include the dihydrofolate reductase gene which is amplified in the presence of methotrexate, and the metallothionein genes which are amplified with heavy metals. In these cases, the nucleotide sequence encoding the polypeptide would be operably linked with the regulatory sequence.

25

Expression Vectors

The present invention also relates to recombinant expression vectors comprising the nucleic acid construct of the invention. The various nucleotide and control sequences described above may be joined together to produce a recombinant expression vector which 30 may include one or more convenient restriction sites to allow for insertion or substitution of the nucleotide sequence encoding the polypeptide at such sites. Alternatively, the nucleotide sequence of the present invention may be expressed by inserting the nucleotide sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the 35 coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (e.g., a plasmid or virus) which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleotide sequence. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The 5 vectors may be linear or closed circular plasmids.

The vector may be an autonomously replicating vector, *i.e.*, a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication; *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome.

10 The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon may 15 be used.

The vectors of the present invention preferably contain one or more selectable markers which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

20 Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in a filamentous fungal host cell include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hygB* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenyltransferase), *trpC* (anthranilate 25 synthase), as well as equivalents thereof.

Preferred for use in an *Aspergillus* cell are the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*.

30 The vectors of the present invention preferably contain an element(s) that permits stable integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

For integration into the host cell genome, the vector may rely on the nucleotide sequence encoding the polypeptide or any other element of the vector for stable integration of the vector into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences for directing integration 35 by homologous recombination into the genome of the host cell. The additional nucleotide sequences enable the vector to be integrated into the host cell genome at a precise

location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleotides, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and most preferably 800 to 1,500 base pairs, which are highly homologous with the corresponding 5 target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleotide sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

10 For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6. The origin of replication may be one having a mutation which makes its functioning temperature-sensitive 15 in the host cell (see, e.g., Ehrlich, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1433).

More than one copy of a nucleotide sequence of the present invention may be inserted into the host cell to increase production of the gene product. An increase in the copy number of the nucleotide sequence can be obtained by integrating at least one 20 additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the nucleotide sequence where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the nucleotide sequence, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

The procedures used to ligate the elements described above to construct the 25 recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

Host Cells

The present invention also relates to recombinant a host cell comprising the nucleic 30 acid construct of the invention, which are advantageously used in the recombinant production of the polypeptides. A vector comprising a nucleotide sequence of the present invention is introduced into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier.

35 The host cell may be a eukaryote, such as a mammalian, insect, plant, or fungal cell. For cloning purposes the host cell may be a bacterial cell as well, e.g. an *E. coli* cell.

In a preferred embodiment, the host cell is a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as 5 cited in Hawksworth *et al.*, 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth *et al.*, 1995, *supra*).

In a more preferred embodiment, the fungal host cell is a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast 10 may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, F.A., Passmore, S.M., and Davenport, R.R., eds, *Soc. App. Bacteriol. Symposium Series No. 9*, 1980).

In an even more preferred embodiment, the yeast host cell is a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell.

15 In a most preferred embodiment, the yeast host cell is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis* or *Saccharomyces oviformis* cell. In another most preferred embodiment, the yeast host cell is a *Kluyveromyces lactis* cell. In another most preferred embodiment, the yeast host cell is a *Yarrowia lipolytica* cell.

20 In another more preferred embodiment, the fungal host cell is a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon 25 catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

In an even more preferred embodiment, the filamentous fungal host cell is a cell of a species of, but not limited to, *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, 30 *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, or *Trichoderma*.

In a most preferred embodiment, the filamentous fungal host cell is an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or *Aspergillus oryzae* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium*

sambucinum, *Fusarium sارochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, or *Fusarium venenatum* cell. In an even most preferred embodiment, the filamentous fungal parent cell is a *Fusarium venenatum* (Nirenberg sp. nov.) cell. In another most preferred embodiment, the filamentous fungal host 5 cell is a *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, 10 transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81: 1470-1474. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156 and WO 96/00787. Yeast may be transformed using the 15 procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; and Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920.

20 Methods of Production

The present invention also relates to methods for producing a polypeptide of the present invention comprising (a) cultivating a strain, which in its wild-type form is capable of producing the polypeptide; and (b) recovering the polypeptide. Preferably, the strain is of the genus *Aspergillus*, and more preferably *Aspergillus oryzae*.

25 The present invention also relates to methods for producing a polypeptide of the present invention comprising (a) cultivating a host cell under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. 30 For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using 35 procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type

Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The polypeptides may be detected using methods known in the art that are specific 5 for the polypeptides. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide as described herein.

The resulting polypeptide may be recovered by methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional 10 procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

The polypeptides of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., 15 preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

Compositions

20 In a still further aspect, the present invention relates to compositions comprising a polypeptide of the present invention.

The composition may comprise a polypeptide of the invention as the major enzymatic component, e.g., a mono-component composition. Alternatively, the composition may comprise multiple enzymatic activities, such as an aminopeptidase, amylase, 25 carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, haloperoxidase, invertase, laccase, lipase, mannosidase, oxidase, pectinolytic enzyme, peptidoglutaminase, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, or 30 xylanase.

The compositions may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry composition. For instance, the polypeptide composition may be in the form of a granulate or a microgranulate. The polypeptide to be included in the composition may be stabilized in accordance with methods known in the art.

35 Examples are given below of preferred uses of the polypeptide compositions of the invention. The dosage of the polypeptide composition of the invention and other conditions

under which the composition is used may be determined on the basis of methods known in the art.

Biofilms

5 Microorganisms growing in biofilms are less susceptible to all types of antimicrobial agents than the same microorganisms when grown in conventional suspension cultures.

It is well known that starved bacteria can be much less susceptible to a variety of antimicrobial challenges. For example, a number of classical antibiotics such as penicillin, perform poorly in slow or non dividing bacteria. Because lysozyme attacks and destroys the

10 peptidoglycan layer regardless of the growth state of the bacteria, it remains effective.

Biofilm control; example dental water lines:

Biofilm buildup within a dental water line can contain biofilms consisting of , Psuedomonas aeruginosa, Proteus mirabilis, Leigonella sp. to name but a few. There is also the possibility

15 of colonisation of species generally found within the oral cavity as a result of the failure of anti retraction valves within the system. The risk of cross infection becomes even more of a potential risk of course when immuno – compromised patients are involved and in this day and age the numbers of patients within this category continues to steadily increase. The need exists for effective control of bacterial biofilm accumulation in dental water lines. A

20 review of biofilms can be found: Watnick P and Kolter R. (2000) Biofilm, city of microbes.

J Bacteriol.;182(10):2675-9.

A typical example of a commercial throat lozenge product is Lysopaine produced by:

BOEHRINGER INGELHEIM FRANCE

25 Active ingredients:

BACITRACIN 200 U.I.

(to 65 iu/mg)

PAPAIN 2 mg

30 to 30 NK / mg

LYSOZYME CHLORHYDRATE 5 mg

to 26000 U FIP / mg : units determined by measuring OD kinetics of lysis of bacteria suspended in buffer. The unit determination was measured by lysis induced change in turbidity of a bacterial culture suspended in buffer.

35

Non active ingredients:

SACCHARIN excipient

MAGNESIUM STEARATE excipient

Menthol aromatisant

5 SORBITOL excipient

For local treatment of point infections limited to the buccal membranes of the oropharynx.

Caution, if clinical indications of a general bacterial infection are evident, antibiotic therapy is advised.

10 Toothpaste:

Lysozyme can be used alone or in combination with other enzymes or even antimicrobial peptides. Examples of other enzymes: Glucose oxidase, lactoperoxidase

A typical toothpaste composition including lysozyme is "Biotene" by Laclede, Inc.

15 2030 East University Drive, Rancho Dominguez, CA 90220, USA

Ingredients

Active Ingredients

Contains: Lactoperoxidase (100gm)

20 Inactive Ingredients

Glucose Oxidase, Lysozyme, Sodium Monofluorophosphate, Sorbitol, Glycerin, Calcium Pyrophosphate, Hydrated Silica, Zylitol, Cellulose Gum, Flavor, Sodium Benzoate, Beta-d-glucose, Potassium Thiocyanate

25 Detergent Composition

The lysozyme of the invention may be added to and thus become a component of a detergent composition, particularly in a liquid detergent having a pH of 7 or lower.

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition 30 suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations, or be formulated for hand or machine dishwashing operations.

In a specific aspect, the invention provides a detergent additive comprising the lysozyme of the invention. The detergent additive as well as the detergent composition may 35 comprise one or more other enzymes such as a protease, a lipase, a cutinase, an amylase, a

carbohydrase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, an oxidase, e.g., a laccase, and/or a peroxidase.

In general the properties of the chosen enzyme(s) should be compatible with the selected detergent, (i.e. pH-optimum, compatibility with other enzymatic and non-enzymatic 5 ingredients, etc.), and the enzyme(s) should be present in effective amounts.

DNA recombination (shuffling)

The nucleotide sequence of SEQ ID NO:1 may be used in a DNA recombination (or shuffling) process. The new polynucleotide sequences obtained in such a process may. 10 encode new polypeptides having lysozyme activity with improved properties, such as improved stability (storage stability, thermo stability), improved specific activity, improved pH-optimum, and/or improved tolerance towards specific compounds.

Shuffling between two or more homologous input polynucleotides (starting-point polynucleotides) involves fragmenting the polynucleotides and recombining the fragments, to 15 obtain output polynucleotides (i.e. polynucleotides that have been subjected to a shuffling cycle) wherein a number of nucleotide fragments are exchanged in comparison to the input polynucleotides.

DNA recombination or shuffling may be a (partially) random process in which a library of chimeric genes is generated from two or more starting genes. A number of known 20 formats can be used to carry out this shuffling or recombination process.

The process may involve random fragmentation of parental DNA followed by reassembly by PCR to new full-length genes, e.g. as presented in US5605793, US5811238, US5830721, US6117679. In-vitro recombination of genes may be carried out, e.g. as described in US6159687, WO98/41623, US6159688, US5965408, US6153510. The 25 recombination process may take place in vivo in a living cell, e.g. as described in WO 97/07205 and WO 98/28416.

The parental DNA may be fragmented by DNA'se I treatment or by restriction endonuclease digests as described by Kikuchi et al (2000a, Gene 236:159-167). Shuffling of two parents may be done by shuffling single stranded parental DNA of the two parents as 30 described in Kikuchi et al (2000b, Gene 243:133-137).

A particular method of shuffling is to follow the methods described in Crameri et al, 1998, Nature, 391: 288-291 and Ness et al. Nature Biotechnology 17: 893-896. Another format would be the methods described in US 6159687: Examples 1 and 2.

35 The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

Examples.**Example 1.****The cloning of a GH 25 lysozyme from *Trametes cinnabarina***

Trametes cinnabarina (=*Pycnoporus cinnabarinus*) CBS114004, *Polyporaceae*, *Polyporales*,

5 *Agricomycetidae*, *Basidiomycetes*, *Basidiomycota*. The strain was isolated from dead branches of *Castannna mollissima*, collected from Huairou county, Beijing, China, by Wen Ping Wu, Sept 2002. The strain was definitively identified as *Trametes cinnabarina* (*Pycnoporus cinnabarinus*) October 11 2002, by Wenping Wu. The fungal strain was deposited on 17 November at Centraal Bureau voor Schimmelcultures, Uppsalalaan 8, P.O.

10 Box 85167, 3508 AD Utrecht, The Netherlands, under the accession number CBS114004.

Fermentation and enzyme fingerprinting:

The fungal strain CBS114004 was fermented in two medias; FG4 and MEX-1 media at 25 degrees C, 160 rpm for 6 days. Culture fluids were tested for enzyme activity assay by 15 standard enzyme fingerprinting assay: (Enzyme fingerprinting of culture fluids.

Enzyme fingerprinting: An enzyme activity profile was obtained by assaying the culture broth on a wide spectrum of enzyme assays. 96 wells microtitre (MT) plates are prepared with substrates and stored at + 10°C until use. Two different pH varieties are prepared: pH3 and pH7. Following substrates are used: 0.05% AZCL (Mazurine dyed and cross-linked substrates, 20 Megazyme) – Amylose, Arabinan, Beta Glucan (Barley), Casein, Collagen, Curdlan, Dextran, Galactan (potato), Galactomannan (Carob), He-Cellulose, Pullulan, Xylan (oat), and Xyloglucan (AZCL-casein can not be used at pH3, and is therefore left out from these plates). Preparation of substrates: pH3 plates: 0.1 g of each AZCL substrate is dissolved in 100 ml 0.2M Succinic acid pH3 + 10 ul TritonX-100 (0.01%), to give a final concentration of 25 0,1% AZCL. pH7 plates: 0.1 g of each AZCL substrate is dissolved in 50 ml sterile H2O plus 10 ul TritonX-100 (0.01%). 50 ml 0.4M MOPS pH 7 is added to each 50 ml AZCL substrate, to give a final volume of 100 ml and a final concentration of 0.2M buffer, 0,1% AZCL.

Laccase and lipase activity assays are included and prepared as follows:

Laccase: 35 ml 0.08 mg/ml Chicago Sky Blue in 0.2 M phosphate/borate-buffer, pH 9 Lipase: 30 A polyvinyl alcohol (PVA)/soybean oil emulsion is prepared by mixing a 2% PVA solution with soybean oil 3:1. Emulsify the oil using ULTRA-TURRAX and mix 12 ml of the emulsion with 500 ml 0.2M sodium-acetate buffer including 10mM CaCl2 pH 5.5 and 5 ml 0.2% Crystal Violet solution. The MT plates are prepared using a Multidrop S20 Stacker, Titertek Instruments, Inc., Alabama, and US. Sterilin 96-wells MT plates are used. 200 µl of each 35 AZCL-substrate and of the lipase substrate and 150 µl of the laccase substrate are dispensed into MT wells and 30-50 µl culture broth are added to each substrate and

incubated over night at 26 degree Celsius. Result scores are made in the assays as follows:
0: no activity, 1: weak activity, 2: strong activity. The mycelium was harvested at 6 days, when the sample showed many different enzyme activities and stored under -80 degrees until used for cDNA library construction.

5

Total RNA isolation:

A Modified Fenozol total RNA extraction method (Active Motif, Inc) was used.

Materials:

10 Fenozol from Active motif Cat. nr. 2100

RNAse free eppendorf tubes, tips

RNase free Chloroform

RNase free isopropanol

RNase free 96% and 70% EtOH

15 RNase free 3 M NAOAc pH 5,2

Phenol-chloroform-mix

CIA(Chloroform:isoamylalcohol, 24:1)

20 The following material was baked at 250°C for 12 hours:

quartz sand

metal spoons

morter and pestle

150ml glass beakers

25

20ml Fenozol in a 150 ml baked glass beaker. Preserved mycelia (-80°C) was ground with baked quartz sand in a pre chilled mortar and pestle under liquid nitrogen. The ground material was transferred with a spoon to the beaker and mixed rapidly to dissolve the frozen material into a thick suspension. The beaker containing the material was transferred to a 50

30°C water bath for 15 minutes. The material was then transferred to an Oakridge tube and 5 ml "RNase free" chloroform was added. The sample was then vortexed vigorously and then allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000g, room temperature for 20 minutes and then the top phase was transferred to a new Oakridge tube. An equal volume of phenol- chloroform mixture was added and the sample was then 35 vortexed vigorously and centrifuged under the same conditions. The sample (top phase was transferred to a new tube and add an equal volume of CIA was added and the vortex and

centrifugation step repeated but with only a 10 minute centrifugation. The aqueous phase was transferred to a new tube and 6.25 ml isopropanol was added, mixed and the material was then incubated at room temperature for 15 minutes. The tubes were then centrifuged at 12,000g for 30 minutes, 4°C. The supernatant was carefully removed and 18ml of 70%
5 ethanol was carefully added. The tubes were centrifuged for 5 minutes,
4°C at 12,000g and then the supernatant was removed and the pellet air dried. The RNA pellet was re-suspended in 500µl DEPC (Dimethylpyrocarbonate)treated water. Heating at 65°C for 10 minutes to aided in re-suspension. The total RNA was stored at- 80°C until further use.

10

PolyA enriched RNA production:

The mTrap Midi mRNA isolation kit from Active Motif was used (Active Motif, Europe). The polyA enriched RNA was purified according to the manufacturer's instruction with the following modification: the total RNA suspension isolated as described above, was used as
15 starting material. 15 ml of the lysis buffer, without protease, was added to approximately 20 µg of total RNA. The mRNA fractions were eluted by column chromatography as described in the protocol and the pools representing 0.5 kb and larger were chosen for cDNA synthesis.

Creation of a cDNA library:

20 The Smart cDNA construction kit (K1051-1, BD Biosciences) was used with the following modifications: After the second strand cDNA synthesis step, the material was purified by GFX spin chromatography according to the manufacturer's instructions (AP Pharma) and the material eluted in 85µl of deionised H₂O. After Sfil digestion, the treated cDNA was size fractionated by EtBr Gel purification according to the method below:

25 1) Prepare a 0.8% SeaPlaque LMP agarose gel in 1 x TBE (in autoclaved LiChroSolv/HPLC H₂O) + ETBr in gel and buffer. Add loading buffer to 100uls of the Sfil digestion from step D and run at 15 V in a clean electrophoresis cell overnight with DNA markers flanking the sample.

2) On a UV box and long wave UV (360 nm), visualize the cDNA and DNA markers.

30 With a scalpel remove all of the gel below 500 bp and remove the top of the sample wells. Slide the remaining gel down on the cell and pour a 1.5 % Seaplaque EtBr agarose gel above the sample wells.

3) Reverse the polarity of the electrophoresis unit and run the DNA a short distance into the 1.5% agarose gel.

35 4) Perform standard gel excision under long wave UV and GFX purification of the gel fragment.

The sample was ligated into the vector pMHas5 in a standard ligation reaction using T4 DNA ligase (New England Biolabs, Inc), according to the manufacturer's instructions. The pMhas5 plasmid and use of a fungal cDNA library produced in the plasmid is described in patent application WO2003044049. The same methodology was used to identify the Trametes 5 GH25 lysozyme from the SigA4 transposon treated library as described in WO2003044049. Sequence, SEQ ID 1, was identified in the original BLAST hit as having strong similarity to both the GENSEQP:AAW85696 *Streptomyces rutgersensis* glycosyl hydrolase GH 25 and the fungal sequence SWISSPROT:P00721 Chalaropsis sp. GH 25 peptide sequence and a number of other less well annotated *Streptomyces* open reading frames from genome 10 sequencing products (*Streptomyces coelicolor*, Q9FBR0) (See homology matrix Table 1). Based on this information, it was decided to express the Trametes GH 25 candidate in *Aspergillus oryzae*.

Table 1.

15 Homology table of top scoring GH25 hydrolases found in public databanks

	Chalaropsis	NP001276	AAW85696	Q9FBR0
Chalaropsis	100	60	32	40
NP001276		100	40	53
AAW85696			100	45
Q9FBR0				100

AAW85696, *Streptomyces rutgersensis*; Q9FBR0, *Streptomyces coelicolor*; NP001276 the GH25 of the invention; Chalaropsis (SWISSPROT:P00721)

25 Example 2.

Expression of GH25 enzyme from Trametes of the invention:

The Sequence ID no 1 was used to design custom oligo nucleotide primers for use in a PCR reaction to generate an insert encoding the enzyme of the invention, flanked by restriction enzyme sites facilitating cloning into an expression vector.

30

Primers:

Primer 1 (Seq ID No 3), NP1276 EcoRI

5'-GCGGAATTCAACATGAAGCTCTCTACCACAGCTC-3'

Primer 2, (Seq ID No 4), NP1276 NotI

35 5'-ATATCGGCCGCAAAGTTACGAAGCTGCGAATTGTC-3'

The underlined portions are additional DNA sequences added to the primers to incorporate restriction enzyme sites used in cloning.

The GH25 lysozyme encoding sequence was amplified from the above cDNA library (see 5 Example 1 above) in the following manner: 1 microliter of cDNA (approximately 10 nanogram of DNA) was used as template in a PCR reaction with the two primers NP1276EcoRI and NP1276NotI.

NP1276 EcoRI: 5'-GCGGAATTCAACATGAAGCTCTTACCACAGCTC-3' (SEQ ID NO:3)
10 NP1276 NotI: 5'-ATATGCGGCCGCAAAGTTACGAAGCTGCGAATTGTC-3' (SEQ ID NO:4)

10 pmole of each primer were used in a 100 microliter reaction volume. The ProofStart polymerase (Qiagen GMBH) was used in conditions recommended by the 15 supplier. After an initial 10 minute heat treatment at 94 degrees C the conditions were as follows: Denaturation temperature; 94 degrees Celsius-30 seconds, annealing temperature; 55 degrees Celsius-30 seconds, and extension at 72 degrees Celsius for 1 minute. A total of 35 cycles were run.

Aliquots of the PCR reaction were separated on a 1% agarose gel. A single distinct 20 band at the predicted 820 base pairs was seen. The fragment was isolated from the gel by GFX spin chromatography (AB Phama) and digested with EcoRI and NotI which cut in the overhangs introduced by the PCR primers. The digested fragments were isolated and cloned into pXYG1051, an Aspergillus expression plasmid based on the plasmid pMStr46 (see the examples of international patent application WO2003070956). Specifically, the AMA 25 autonomous replication region was removed by HindIII digestion and reigation resulting in an integrative vector. After standard ligation, transformation into DH10B E. coli, colonies that grew under ampicillin selection on LB plates were identified. 10 colonies were chosen in order to prepare plasmid DNA. The resulting plasmid DNA was sequenced using the vector primers of pXYG1051 revealed in patent application WO2003070956. One of the clones that 30 was without PCR errors (NP001276-4) was chosen for a Qiagen midi scale plasmid prep (Qiagen GMBH).

The chosen clone (NP001276-4) was used for expression of the enzyme in Aspergillus (see below) and the EcoRI and NotI insert comprising the gene of the invention was used for subcloning the fragment into a standard bacterial vector, pBlueScript KS+ 35 (Stratagene Inc.), and transformation into the E. coli strain DH10B (from Clontech). This strain was deposited 8 December 2003 at Deutsche Sammlung von Mikroorganismen und

Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany, under the accession number DSM 16084.

Expression of clone NP001276 in Aspergillus:

5 The cDNA clone, NP001276-4, was transformed into *Aspergillus oryzae* strain Jal355 (disclosed in international patent application WO2003070956). 30 transformants were re-isolated twice under selective and non-inducing conditions on Cove nitrate minimal plates with sucrose as the carbon source. To test expression of NP1276, transformants were grown for 4 days at 30 degrees Celsius in tubes with 10 ml YPM (2% peptone, 1% yeast extract, 10 2% maltose). Supernatants were run on NuPage 10% Bis-Tris SDS gels (Invitrogen) as recommended by the manufacturer. The *Aspergillus* strains grew well even when induced for the expression of Plectasin with maltose. A distinct band of the size expected for GH 25 lysozyme (23 kDa) was seen in most transformants, whereas this band was not seen in the untransformed host strain *A. oryzae* BECh2. A single, strongly expressing *Aspergillus* 15 transformant (EXP00787) was chosen for continued study. A large scale shake flask experiment was performed with 20 X 1000ml Erlingmeyer shake flasks with three baffles. Each flask contained 100mls FG4P media (FG4P: 3% Soybean meal (SFK 102-2458), 1.5% Maltodextrin (Roquette), 0.5% Pep-tone bacto (Difco 0118), 1.5% KH₂PO₄ (Merck 4873), 0.2mls/liter Pluronic PE 6100 (BASF)). Conditions used: 30 Degrees C, 150 RPM, 3 days 20 growth. The culture fluid was harvested by filtration through two layers of miracloth and frozen until used.

Example 3.

Purification of GH25 enzyme of the invention:

25 Description of purification:
1.7 l of fermentation broth was germ filtrated and purified on a SP-sepharose column using 50mM NaAc pH 5/ 50mM NaAc, 1M NaCl pH 5. The pooled fractions were concentrated on Amicon cell (Millipore) with a cut off 10kDa. The N-terminal amino acid 30 sequence (EKRANPKGID) confirmed that the purified protein was mature GH25 enzyme.

Example 4.

Detection of lysozyme activity by a cell wall assay.

Lyophilized cells of *Micrococcus lysodeikticus* (Sigma) was used as substrate. The substrate 35 was suspended in a Britton&Robinson buffer (0.08 M phosphoric acid, 0.08 M boric acid and 0.08 M acetic acid; pH adjusted to 2-12 with 1 M NaOH) at the appropriate pH to a final

concentration of 0.3 mg/ml. To each well in a 96 well microtiter plate was added 100 μ l substrate suspension and 30 μ l enzyme solution at a concentration of approximately 1 mg/ml. Changes in turbidity was measured in a microplate reader at 450 nm for 5 minutes after addition of the enzyme. The slope of the curve was calculated and used as a relative 5 measure of enzyme activity.

Temperature curve: The purified enzyme was incubated at 40°, 50°, 60° and 70°C for 15 min. Samples were cooled to RT and the relative residual activity at pH 5 was measured in the above mentioned assay. The enzyme was stable up to 50°C.

10 Table 2.

Temperature, °C	Relative residual activity, %
40	100
50	98
60	64
70	28

pH curve: Substrate suspensions at pH 4, 5, 6, 7 and 8 were prepared, and relative activity of the enzyme was measured as described above. The enzyme has pH optimum at pH 5.

Table 3.

pH	Relative activity, %
4	35
5	100
6	41
7	20
8	15

15

Deposit of Biological Material

The following biological material has been deposited under the terms of the Budapest Treaty with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder 20 Weg 1 B, D-38124 Braunschweig, Germany, and given the following accession number:

Deposit
E. coli DH10B

Accession Number
DSM 16084

Date of Deposit
5 December 2003

The following biological material has been deposited under the terms of the Budapest Treaty with the Centraal Bureau voor Schimmelcultures, Uppsalalaan 8, P.O. Box 85167, 3508 AD Utrecht, The Netherlands, and given the following accession number:

5 Deposit	Accession Number	Date of Deposit
<i>Trametes cinnabarina</i>	CBS 114004	17 November 2003

0-1	Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis) Prepared Using	
0-1-1		PCT-SAFE [EASY mode] Version 3.50 (Build 0002.169)
0-2	International Application No.	PCT/DK 2005/000099
0-3	Applicant's or agent's file reference	10515.204-WO

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	34
1-2	line	23
1-3	Identification of deposit Name of depositary institution	DSMZ DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	05 December 2003 (05.12.2003)
1-3-4	Accession Number	DSMZ 16084
1-5	Designated States for Which Indications are Made	all designations
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
2-1	page	35
2-2	line	06
2-3	Identification of deposit Name of depositary institution	CBS Centraalbureau voor Schimmelcultures
2-3-2	Address of depositary institution	Uppsalaalaan 8, NL-3584 CT Utrecht, The Netherlands / P.O. Box 85167, NL-3508 AD Utrecht, The Netherlands
2-3-3	Date of deposit	17 November 2003 (17.11.2003)
2-3-4	Accession Number	CBS 114004
2-5	Designated States for Which Indications are Made	all designations

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